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Respiratory Elicitors from *Rhizobium meliloti* Affect Intact Alfalfa Roots[1]

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Molecules produced by *Rhizobium meliloti* increase respiration of alfalfa (*Medicago sativa* L.) roots. Maximum respiratory increases, measured either as CO_2 evolution or as O_2 uptake, were elicited in roots of 3-d-old seedlings by 16 h of exposure to living or dead *R. meliloti* cells at densities of 10^7 bacteria/mL. Excising roots after exposure to bacteria and separating them into root-tip- and root-hair-containing segments showed that respiratory increases occurred only in the root-hair region. In such assays, CO_2 production by segments with root hairs increased by as much as 100% in the presence of bacteria. Two partially purified compounds from *R. meliloti* 1021 increased root respiration at very low, possibly picomolar, concentrations. One factor, peak B, resembled known pathogenic elicitors because it produced a rapid (15-min), transitory increase in respiration. A second factor, peak D, was quite different because root respiration increased slowly for 8 h and was maintained at the higher level. These molecules differ from lipo-chitin oligosaccharides active in root nodulation for the following reasons: (a) they do not curl alfalfa root hairs, (b) they are synthesized by bacteria in the absence of known plant inducer molecules, and (c) they are produced by a mutant *R. meliloti* that does not synthesize known lipo-chitin oligosaccharides. The peak-D compound(s) may benefit both symbionts by increasing CO_2, which is required for growth of *R. meliloti*, and possibly by increasing the energy that is available in the plant to form root nodules.

Roots colonized by microorganisms evolve more CO_2 than sterile roots (Barber and Martin, 1976; Meharg and Killham, 1991). However, the source of the extra CO_2 is difficult to determine when both roots and microbes are respiring (Cheng et al., 1993; Swinnen, 1994). It is possible that increases in root-plus-bacterial respiration result when soil microorganisms first enhance root exudation and then respire C compounds in the exudate (Meharg and Killham, 1991). Alternative explanations, however, should also be considered. For example, cell wall fragments from pathogenic fungi increase plant cell respiration (Norman et al., 1994), and it is plausible that products from rhizosphere bacteria may have similar effects. In fact, plant-derived CO_2 may help *Rhizobium* and *Bradyrhizobium* spp. rhizobia colonize roots because they require exogenous CO_2 for growth (Lowe and Evans, 1962). A role for rhizosphere CO_2 in rhizobial growth is supported by the fact that biotin, a cofactor required for using bicarbonate, limits alfalfa (*Medicago sativa* L.) root colonization by *Rhizobium meliloti* (Streit et al., 1996).

Rhizobial bacteria form root nodules on legumes by altering genetic, biochemical, physiological, and morphological characteristics of root cells. Many of these changes occur in response to specific LCO signals produced by rhizobia in the presence of plant signal molecules (Dénarié and Cullimore, 1993; Spaink, 1995). Whereas data show that LCOs alter root flavonoid metabolism before nodules appear (Spaink et al., 1991; Savouré et al., 1994), the effects of LCOs and external rhizobia on primary C metabolism of root cells are poorly understood.

Given the rapidly changing metabolic requirements of plant cells at bacterial infection sites, it would not be surprising to find that plant cells respond to rhizobia by modifying the rate or patterns of primary C metabolism. It is possible, therefore, to determine whether rhizobial products increase plant cell respiration. To explore this hypothesis we searched for extracellular products of *R. meliloti* that enhance root respiration in alfalfa, their normal host plant.

MATERIALS AND METHODS

Plant Growth and Inoculation

Seeds of alfalfa (*Medicago sativa* L. cv Moapa 69) were surface sterilized for 15 min in 70% ethanol, rinsed with water, and allowed to imbibe for 4 h with aeration before germinating in a hydroponic system (Maxwell et al., 1989) containing N-free nutrient solution (DeJong and Phillips, 1981). Each 400-mL plastic box contained 1 g of seeds and produced about 400 seedlings after being maintained in a sterile manner for 3 d with aeration at 25°C under indirect sunlight supplemented with fluorescent lights. Plants used in these experiments consisted of cotyledons and roots with an occasional primary leaf.

Rhizobium meliloti 1021 (Rm1021) (Meade et al., 1982) and *R. meliloti* TJ1A3 (Rm1021*nodC*::Tn5) (Jacobs et al., 1985) were grown to the early stationary phase in a defined minimal medium (Vincent, 1970). *Agrobacterium tumefaciens* 1D1609 (Palumbo, 1997) and *Escherichia coli* S17-1 (Simon et al., 1983) were grown in AB mineral medium with 0.5%

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Abbreviations: CFU, colony-forming units; LCO(s), lipo-chitin oligosaccharide(s); Nod, nodulation.

(w/v) sodium succinate (Cangelosi et al., 1991) and Luria-Bertani medium (Sambrook et al., 1989), respectively. Bacteria were collected by centrifugation and washed three times with sterile water before roots were inoculated. UV irradiance for killing cells in some experiments was supplied as a 25-min treatment with a transilluminator (model T1202, Sigma). The absence of living cells in UV-killed cultures and sterile, noninoculated control treatments was verified by plating on tryptone yeast medium (Beringer, 1974) for *R. meliloti* or Luria-Bertani medium for *A. tumefaciens* and *E. coli*.

Experiments used 5 × 10^7 CFU of bacteria per mL of plant nutrient solution unless otherwise noted. Bacteria were grown in their respective media, washed twice in sterile water, suspended in 1 mL of water, and added to the plant nutrient solution of alfalfa seedlings 3 d after germination, when roots were approximately 4 cm long. Sterile water (1 mL) was added to the sterile, noninoculated controls. Plants were harvested to measure root respiration at the times indicated in various experiments. At harvest, roots were excised, blotted briefly onto a paper towel, weighed, and enclosed in a 10-mL gas-tight test tube. Each replicate contained 1 g fresh weight of roots from about 200 plants; every experiment had three or four replicates and all experiments were repeated at least twice.

Analyses

Changes in CO_2, and in some experiments O_2, were measured at 45°C with a thermistor detector on a Sigma 4 gas chromatograph (Perkin-Elmer) equipped with a column (3.05 m × 3.2 mm) containing Chromosorb 102 for CO_2 and Molecular Sieve 5A for O_2. He was used as the carrier gas at flow rates of 15 cm^3/min for O_2 and 35 cm^3/min for CO_2. The change in gas composition during the first 30 min after sealing assay tubes was used to calculate respiration rates. Data were analyzed with standard statistical methods to determine SE or $LSD_{0.05}$ values for comparisons of treatment effects by a Student's *t* test or analysis of variance (Steel and Torrie, 1960).

Supernatant from the bacterial growth medium was collected by centrifugation and treated for 4 h with SM-2 Bio-Beads (30 g/L) (Bio-Rad). Compounds adsorbing to the Bio-Beads were eluted with methanol (10 mL/g) and dried under a vacuum. Samples for HPLC were dissolved in water, injected into a HPLC system (Waters) fitted with a Lichrosorb RP-18 column (250 × 4.6 mm) (Alltech Associates, Inc., Deerfield, IL), and eluted with water at 0.5 mL/min from 0 to 10 min. From 10 to 70 min a linear gradient increasing to 100% methanol was applied, and the analysis continued isocratically in 100% methanol for another 20 min. Eluting compounds were monitored with a photodiode array detector (model 996, Waters). Eluate was collected on a Cygnet fraction collector (ISCO, Inc., Lincoln, NE) every minute and dried by lyophilization.

Root-hair-curling capacity of various fractions was assayed by exposing root hairs of 3-d-old alfalfa seedlings grown on water agar. Test compounds were added in 100-μL drops to the root hair zone, and roots were monitored for 2 d with a light microscope at 100× magnification to detect morphological changes.

RESULTS

Bacterial Enhancement of Root Respiration

Initial experiments in which Rm1021 bacteria were applied to roots of 3-d-old alfalfa seedlings established that soon after 4 h, root respiration increased significantly ($P \leq 0.05$) relative to sterile, noninoculated controls (Fig. 1). In various experiments the promotive effect reached a maximum 8 to 12 h after inoculation, and remained at high levels for at least 24 h. On the basis of these results, roots in subsequent experiments were assayed for CO_2 evolution 16 to 24 h after inoculation. Germinating seedlings in the presence of 8 mM NH_4NO_3 had no effect on these results (data not shown), and all experiments reported here were done under N-free nutrient conditions where root exudates have been characterized (Maxwell et al., 1989).

Tests showed that CO_2 evolution by roots in this experimental system was linear for more than 1 h after excision (data not given). For that reason all assays reported here were conducted for 30 min immediately after excision. In several initial experiments in which both CO_2 evolution and O_2 uptake were measured, the CO_2 evolution increased in proportion to O_2 uptake (data not shown). Subsequent experiments measured only CO_2 evolution as an indicator of respiration.

Living bacteria were not required for the respiratory response because UV-killed cells also increased CO_2 evolution by the roots (Fig. 2). In fact, dead bacteria elicited significantly higher rates of root respiration than living cells in several, but not all, experiments. Under the conditions of these assays, an alfalfa pathogen, *A. tumefaciens* 1D1609, elicited a respiratory response very similar to that

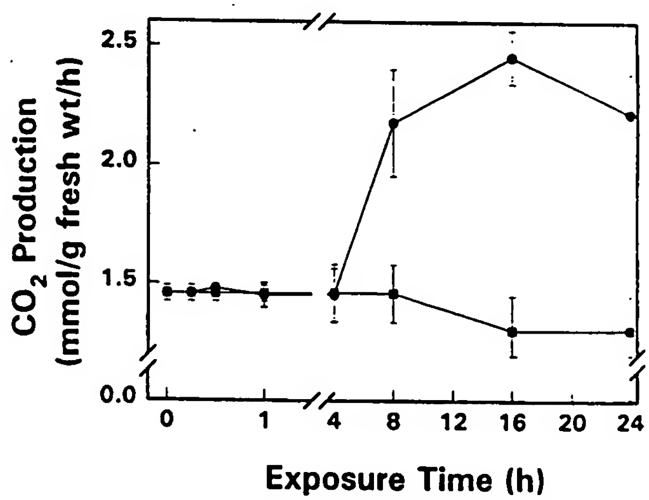

Figure 1. Enhancement of alfalfa root respiration by *R. meliloti* 1021. Bacteria were supplied to 3-d-old seedling roots at time 0, and 4-cm primary roots, including the tip, were excised at various times to measure respiration. Values are means ± SE from three replicates maintained as a sterile, noninoculated control (■) or treated with Rm1021 (●). fresh wt, Fresh weight.

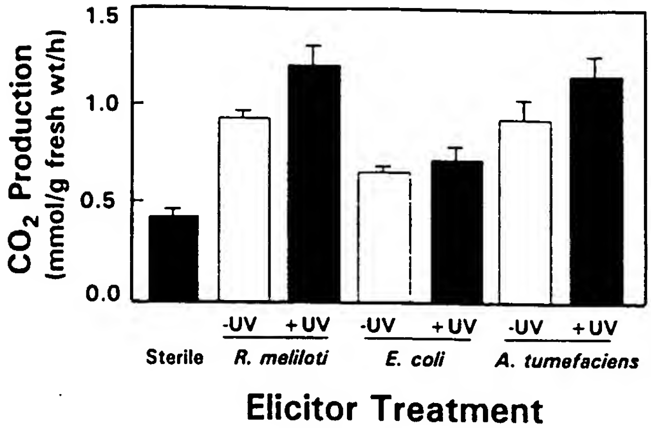

Figure 2. Effects of various bacterial species on respiration of alfalfa roots. Roots of 3-d-old seedlings were exposed to living (−UV) or dead (+UV) bacterial cells for 20 h, then 4-cm primary roots, including tips, were excised to measure respiration. Values are means + SE from three replicates. fresh wt, Fresh weight.

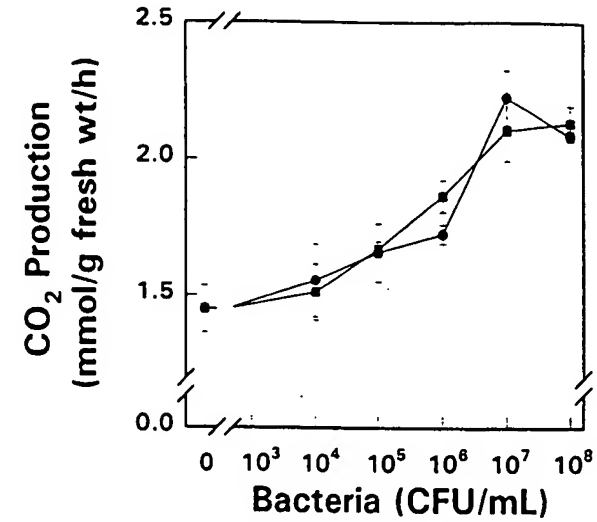

Figure 3. Effects of bacterial concentration on respiration of alfalfa roots. Roots of 3-d-old seedlings were exposed to living ( ) or UV-killed (■) Rm1021 cells for 16 h, then root segments bearing root hairs were excised to measure respiration. Values are means ± SE from three replicates. fresh wt, Fresh weight.

obtained with *R. meliloti* (Fig. 2). *E. coli* produced significant, but less marked, increases in CO_2 evolution by the roots.

Tests showed clearly that the respiratory enhancement by Rm1021 occurred in the root-hair region (Table I). In those experiments, bacteria were exposed to the intact plant and then roots were excised and divided into two sections, a 1-cm tip and a 3-cm subtending segment, which had differentiated root hairs by d 3. Although root tips had a much higher rate of CO_2 evolution, Rm1021 enhanced respiration only in the root-hair zone. Calculations made by summing CO_2 produced from the two segments indicated that cutting the roots did not increase respiration markedly. On the basis of these results, all subsequent assays were conducted by exposing bacteria or elicitor fractions to the intact plant and then measuring respiration only in the root segments bearing root hairs.

Treatments in which different numbers of Rm1021 cells were inoculated onto roots showed that at least 10^7 CFU/mL were required for the maximum response (Fig. 3). This concentration of cells was visible to the naked eye. No common contaminants with especially powerful elicitor activity were detected. For example, in a few cases in which plant-growth containers were purposely left open to the air for 24 h, low numbers of air-borne bacteria were detected (e.g. 10^4 CFU/mL), but root respiration was similar to the insignificant response produced by comparable numbers of Rm1021 cells (data not shown).

The well-characterized LCOs from *R. meliloti*, which function as Nod factors, were not required for the respiratory response studied in these experiments (Fig. 4). Mutant *R. meliloti* strain TJ1A3, which produces neither Nod-factor LCOs nor root nodules, was fully capable of eliciting increased respiration in alfalfa root segments bearing root hairs after 16 h of exposure to the intact seedling.

Respiration values measured in sterile root segments bearing root hairs differed somewhat in various experiments (compare Figs. 3 and 4). In all cases, however, rhizobial cells or elicitor molecules enhanced significantly the basal rate of respiration. In most instances, elicitors increased respiration 80 to 100% over the sterile, noninoculated control value. No attempt was made to relate these differences between experiments to possible changes in

Table I. *R. meliloti effects on alfalfa root respiration*

Intact seedlings were exposed to bacteria for 24 h, then roots were cut into a 1-cm tip and a 3-cm root-hair-bearing region to measure CO_2 production. Values in a column followed by different letters show significant ($P \leq 0.01$) treatment effects. Roots exposed to dead Rm1021 cells remained sterile throughout the 24-h incubation period.

Treatment	Segment with Root Hairs	Root Tip	Entire Root	
			Uncut control	Cut root[a]
	mmol CO_2 evolved g^{-1} fresh wt h^{-1}			
Sterile control	0.66a	3.2a	1.10a	1.17a
Living Rm1021	1.26b	3.0a	1.54b	1.60b
UV-killed Rm1021	1.35b	3.3a	1.65b	1.74b

[a] Calculated by summing data from cut segments.

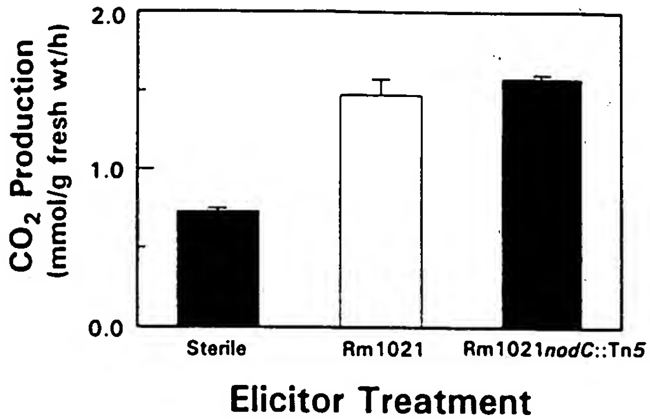

Figure 4. Promotion of alfalfa root respiration by a nonnodulating *R. meliloti* mutant. Roots of 3-d-old seedlings were exposed to wild-type Rm1021 or mutant Rm1021*nodC*::Tn*5* cells for 16 h, then root segments bearing root hairs were excised to measure respiration. Values are means ± SE from three replicates. fresh wt, Fresh weight.

external or internal factors (e.g. root aeration or circadian rhythms).

Isolation of Bacterial Factors Enhancing Root Respiration

Supernatant from dense (5×10^9 CFU/mL) Rm1021 cultures contained elicitor activity that was completely removed by adsorption to Bio-Beads. In a typical experiment, in which sterile root-hair-bearing root segments respired 0.92 ± 0.15 mmol CO_2 g^{-1} fresh weight h^{-1} (mean ± SE) and living Rm1021 bacterial cells elicited 1.48 ± 0.15 mmol CO_2 g^{-1} fresh weight h^{-1}, the bacterial products that adsorbed to Bio-Beads elicited 1.57 ± 0.27 mmol CO_2 g^{-1} fresh weight h^{-1}. Culture supernatant remaining after the Bio-Bead treatment elicited respiration of 0.81 ± 0.15 mmol CO_2 g^{-1} fresh weight h^{-1}. All subsequent work was done with the fraction that adsorbed to Bio-Beads and was eluted with methanol. In developing this purification procedure, fractions were tested for elicitor activity at concentrations 10-fold higher than the minimum required to detect root-hair-curling activity in culture filtrates from luteolin-treated bacteria. Because tests with 1000-fold higher concentrations detected traces of elicitor activity that had not adsorbed to Bio-Beads, this method probably purified more than 99% of the elicitor molecules away from numerous polysaccharides present in the culture filtrate.

HPLC analysis of the lipophilic (i.e. Bio-Bead-binding) fraction from culture supernatant of Rm1021 cells indicated that four major peaks (A, B, C, and D) were present (Fig. 5). Tests proved that peak A had no effect on root respiration and did not curl root hairs (data not shown). Peak C was present only in culture filtrates of cells exposed to the known *nod* gene inducer luteolin (Fig. 5A), and it curled root hairs (data not shown). Those facts suggested that peak C contained Nod-factor LCOs, and no further experiments were done with that fraction. Material in peaks B and D did not curl alfalfa root hairs in tests using c ncentrations that were normalized through bacterial numbers to those present in the root hydroponic system (data not shown). Rm1021 cells grown without luteolin produced compounds in peaks B and D (Fig. 5B), and each liter of culture filtrate yielded approximately 5 mg of peak B and 1 mg of peak D. No HPLC peaks resembling peak B in terms of retention time and UV-visible spectrum were found in supernatant from either *E. coli* or *A. tumefaciens*. A minor peak with a UV-visible spectrum similar to peak D but a different retention time was present in supernatant from *A. tumefaciens* but not from *E. coli*.

Very small amounts of the material in peaks B and D increased root respiration after intact seedlings had been treated for 16 h (Fig. 6). Peak-D material, for example, promoted respiration significantly ($P \leq 0.05$) at 6.7×10^{-10} g/L, and a 10-fold higher concentration produced a one-half-maximum response. The maximum increase in respiration elicited by peak D was consistently twice that produced by peak B after 16 h of treatment.

Experiments with these HPLC fractions showed that peaks B and D differed greatly in the time required for changes in root respiration to occur (Fig. 7). Peak B produced a rapid increase in respiration within 15 min, which declined over the next 20 h, whereas peak D required 8 h to elicit a maximum response, which was maintained until the end of the 20-h experiment.

DISCUSSION

Results from this study establish that two soil bacteria, *R. meliloti* and *A. tumefaciens*, increase respiration of alfalfa roots to a greater extent than *E. coli*. Although it has been shown previously that roots colonized by microorganisms evolve more CO_2 than sterile roots (Barber and Martin, 1976; Meharg and Killham, 1991), data presented here prove that bacterial respiration of root exudates is not required for that response. Both dead bacteria (Fig. 2) and partially purified rhizobial products (Figs. 6 and 7) en-

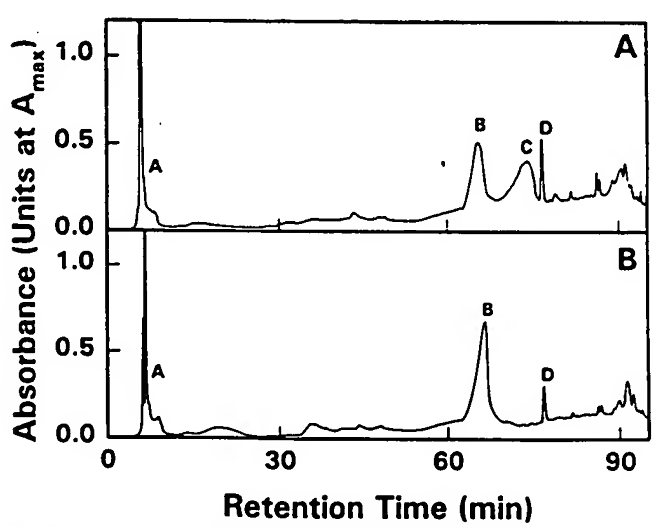

Figure 5. HPLC analyses of culture filtrates from *R. meliloti* 1021. Cells were grown with (A) or without (B) 3 mM luteolin, an inducer of genes required for production of Nod-factor LCOs contained in peak C. Compounds were eluted from a C_{18} column with an increasing concentration of methanol.

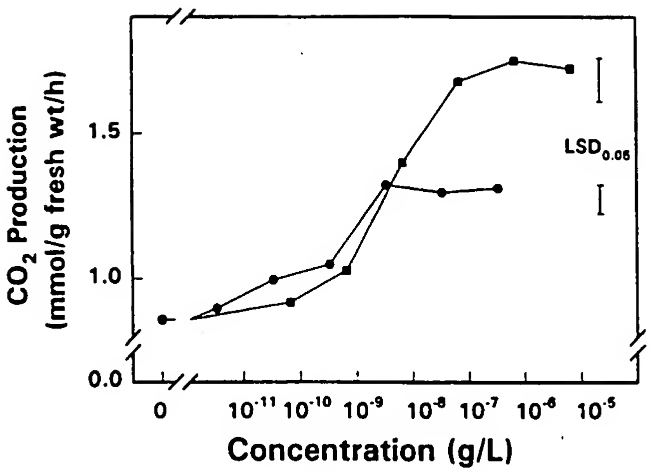

Figure 6. Effects of partially purified bacterial products on alfalfa root respiration. Material from peak B (●) and peak D (■) from Rm1021 culture medium (Fig. 5B) was supplied to roots of 3-d-old seedlings at the indicated concentrations. Respiration of root segments bearing root hairs was measured 16 h later. Values are means from two replicates, each containing roots of 200 plants. fresh wt, Fresh weight.

hanced alfalfa root respiration. Important characteristics of this bacterial elicitation of alfalfa root respiration were: (a) localization of the response in the region containing root hairs (Table I) and (b) the extreme sensitivity of the response (Fig. 6). If the active material in peak D has a molecular weight between 500 and 1000, then it produces a one-half-maximum increase in root respiration at a concentration of 10 to 20 pM. Clearly, two different types of factors are produced by rhizobia because one, HPLC peak B, elicits a rapid (15-min) increase in respiration, whereas the other, peak D, requires a longer (8-h) period for the plant response (Fig. 7). Material in peak D may be the more important of these two fractions because the time course of the plant response to the purified material is similar to that observed for intact cells (compare Figs. 7 and 1). In addition, a compound spectrally similar to peak D was produced by *A. tumefaciens*, a rhizosphere bacterium, but not by *E. coli*.

The elicitor molecules found in this study differ from known rhizobial LCOs involved in root nodule formation (Dénarié and Cullimore, 1993; Spaink, 1995). Unlike those Nod factors, molecules studied here are still produced by Rm1021*nodC*::Tn*5*, a *nodC* mutant (Fig. 4), and they are synthesized by Rm1021 cells grown in the absence of *nod*-gene-inducing compounds (Fig. 5B). Tests showed that, unlike Nod-factor LCOs, compounds present in peaks B and D (Fig. 5) did not curl alfalfa root hairs. Because rhizobia inoculated on plants in these experiments were grown in the absence of *nod* gene inducers, Nod factors were not present initially in the hydroponic rooting medium. Inducible, *nod*-gene-dependent compounds, such as Nod-factor LCOs that are present in HPLC peak C, may have been synthesized by Rm1021 cells during the 16- to 24-h incubation with intact roots, because alfalfa roots release *nod*-gene-inducing compounds under these experimental conditions (Maxwell et al., 1989). However, the magnitude of the response obtained with Rm1021*nodC*::Tn*5* (Fig. 4) proves that any Nod factors produced by bacteria during exposure to these roots did not function additively with the respiratory elicitors studied here. Moreover, the capacity of UV-killed bacteria to induce the phenomena (Figs. 2 and 3) reinforces the concept that constitutive products, rather than plant-induced bacterial products, are fully capable of eliciting the respiratory response.

Cell wall fragments from pathogenic fungi can elicit increased respiration in plant cells. For example, parsley cell cultures treated with a *Phytophthora megasperma* elicitor fraction increased respiration within 20 min (Norman et al., 1994). Rhizobial material in HPLC peak B elicited a similar and rapid response (Fig. 7), and rhizobia defective in surface polysaccharides are known to induce a defense-like response (Niehaus et al., 1993). Although micromolar concentrations of Nod factor induce the accumulation of defense-related transcripts in alfalfa roots (Savouré et al., 1997), peak B is not a Nod factor because it was produced in the absence of luteolin and has no root-hair-curling activity. Respiratory increases produced by intact rhizobial cells (Fig. 1) and by material in the HPLC peak D (Fig. 7) required a much longer period to develop. This agrees with the concept that *R. meliloti* does not elicit the classic host defense response in alfalfa roots (McKhann and Hirsch, 1994). It is not known if material in peak B reached seedling roots in our experiments with intact bacterial cells, but peak D, which has a characteristic UV-visible spectrum, was found in HPLC analyses of the hydroponic root solution after 20 h of exposure to intact line Rm1021 cells (data not shown).

R. meliloti may derive several important benefits from increasing alfalfa root respiration. First, because bacteria use CO_2 for growth in reactions such as acetyl-CoA car-

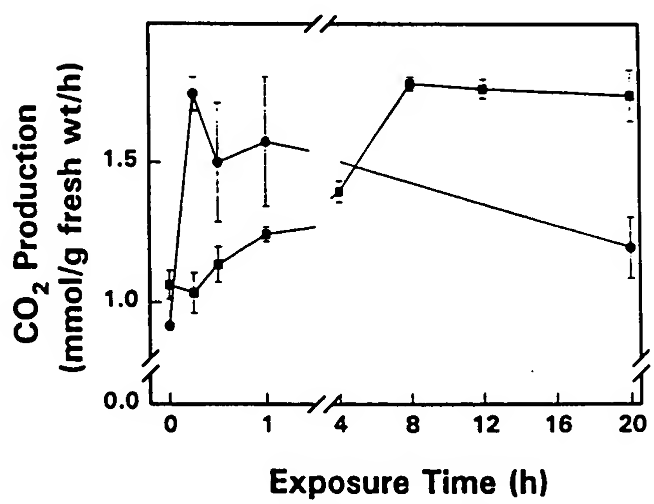

Figure 7. Short-term respiratory responses in alfalfa root segments. Material from peak B ( ) or peak D (■) in Rm1021 culture medium (Fig. 5B) was applied at 10^{-6} g/L to intact seedling roots for the indicated period before respiration of root-hair-c ntaining segments was measured.

boxylation (Burns et al., 1995) and pyruvate carboxylation (Dunn et al., 1996), rhizobia could use plant-derived CO_2 directly and thereby conserve other root-exudate compounds (e.g. amino acids) for direct incorporation into bacterial protoplasm (Phillips and Streit, 1996). Second, the quantity of other compounds in root exudates may increase in conjunction with the enhanced root respiration. Levels of certain flavonoids in alfalfa root exudate increase in the presence of *R. meliloti* cells (Dakora et al., 1993), and that response can be produced by Nod-factor LCOs in other legumes (Spaink et al., 1991; Savouré et al., 1997), possibly by depolarizing the root-hair cell membrane (Ehrhardt et al., 1992). Whether rhizobial elicitors in peaks B and D affect root exudation in addition to respiration is not known.

The mechanism underlying rhizobial elicitation of increased root respiration remains to be defined. Because CO_2 evolution increased in proportion to O_2 uptake in these experiments, root respiration rather than decarboxylation was the source of the CO_2. The response measured here in root-hair-bearing segments may herald changes in primary C metabolism that develop later in root nodules (Vance and Heichel, 1991; Werner, 1992), but any such relationship remains to be demonstrated. Plants treated with Rm1021 required 48 h before root-hair deformation occurred (data not shown), so energy derived from increased respiration after 24 h may have contributed to that process. The source of additional respiratory substrate cannot be determined from these experiments, but several different processes may operate. Short-term responses (<60 min), such as the effect of material in peak B, may involve oxidation of sugar from known flavonoid glycosides (Tiller et al., 1994), which presumably are present in cortical cell vacuoles. Longer-term responses (4–8 h) could involve changes in photosynthate partitioning or possibly even an increase in photosynthesis. As the elicitor molecules in peaks B and D are identified, they will be important reagents for defining the poorly understood transduction systems affecting root respiration.

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